## REMARKS

Entry of the amendments to the claims before examination of the application is respectfully requested.

If there are any questions regarding this Preliminary Amendment or this application in general, a telephone call to the undersigned would be appreciated since this should expedite the prosecution of the application for all concerned.

Respectfully submitted,

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# ~~GROWTH FACTOR~~ VASCULAR ENDOTHELIAL GROWTH FACTOR D (VEGF-D) ANTIBODIES AND VECTORS, AND METHODS OF USE

## CROSS REFERENCE TO RELATED APPLICATIONS

**[0001]** This application is a division of co-pending application Serial No. 09/296,275, filed April 22, 1999, which is a division of application Serial No. 08/915,795, filed August 21, 1997, now U.S. Patent No. 6,235,713. This application also claims the benefit of the filing dates of the following copending U.S. Provisional Applications: Serial No. 60/023,751, filed August 23, 1996; Serial No. 60/031,097, filed November 14, 1996; Serial No. 60/038,814, filed February 10, 1997; and Serial No. 60/051,426, filed July 1, 1997.

## FIELD OF THE INVENTION

**[0002]** This invention relates to growth factors for endothelial cells, and in particular to a novel vascular endothelial growth factor, DNA encoding the factor, and to pharmaceutical and diagnostic compositions and methods ~~utilising~~ utilizing or derived from the factor.

## BACKGROUND OF THE INVENTION

**[0003]** Angiogenesis is a fundamental process required for normal growth and development of tissues, and involves the proliferation of new capillaries from pre-existing blood vessels. Angiogenesis is not only involved in embryonic development and normal tissue growth, repair, and regeneration, but is also involved in the female reproductive cycle, establishment and maintenance of pregnancy, and in repair of wounds and fractures. In addition to angiogenesis which takes place in the normal individual, angiogenic events are involved in a number of pathological processes, notably ~~tumour~~

tumor growth and metastasis, and other conditions in which blood vessel proliferation, especially of the microvascular system, is increased, such as diabetic retinopathy, psoriasis andarthropathies. Inhibition of angiogenesis is useful in preventing or alleviating these pathological processes.

**[0004]** On the other hand, promotion of angiogenesis is desirable in situations where vascularization is to be established or extended, for example after tissue or organ transplantation, or to stimulate establishment of collateral circulation in tissue infarction or arterial stenosis, such as in coronary heart disease and thromboangitis obliterans.

**[0005]** Because of the crucial role of angiogenesis in so many physiological and pathological processes, factors involved in the control of angiogenesis have been intensively investigated. A number of growth factors have been shown to be involved in the regulation of angiogenesis; these include fibroblast growth factors (FGFs), platelet-derived growth factor (PDGF), transforming growth factor α (TGFα), and hepatocyte growth factor (HGF). See, for example, Folkman *et al.*, "Angiogenesis", J. Biol. Chem., 1992 267 10931-10934 for a review.

**[0006]** It has been suggested that a particular family of endothelial cell-specific growth factors and their corresponding receptors is primarily responsible for stimulation of endothelial cell growth and differentiation, and for certain functions of the differentiated cells. These factors are members of the PDGF family, and appear to act via endothelial receptor tyrosine kinases (RTKs). Hitherto four vascular endothelial growth factor subtypes have been identified.

Vascular endothelial growth factor (VEGF), now known as VEGF-A, has been isolated from several sources. VEGF-A shows highly specific mitogenic activity against endothelial cells, and can stimulate the whole sequence of events leading to angiogenesis. In addition, it has strong chemoattractant activity towards monocytes, can induce plasminogen activator and plasminogen activator inhibitor in endothelial cells, and can also influence microvascular permeability. Because of the latter activity, it is also sometimes referred to as vascular permeability factor (VPF). The isolation and properties of VEGF have been reviewed; see Ferrara *et al.*, "The Vascular Endothelial Growth Factor Family of Polypeptides", J. Cellular Biochem., 1991 47 211-218 and Connolly, "Vascular Permeability Factor: A Unique Regulator of Blood Vessel Function", J. Cellular Biochem., 1991 47 219-223.

**[0007]** More recently, three further members of the VEGF family have been identified. These are designated VEGF-B, described in International Patent Application No. PCT/US96/02957 (WO 96/26736) by Ludwig Institute for Cancer Research and The University of Helsinki, VEGF-C, described in Joukov *et al.*, The EMBO Journal, 1996 15 290-298, and VEGF2, described in International Patent Application No. PCT/US94/05291 (WO 95/24473) by Human Genome Sciences, Inc. VEGF-B has closely similar angiogenic and other properties to those of VEGF, but is distributed and expressed in tissues differently from VEGF. In particular, VEGF-B is very strongly expressed in heart, and only weakly in lung, whereas the reverse is the case for VEGF. This suggests that VEGF and VEGF-B, despite the fact that they are co-expressed in many tissues, may have functional differences.

**[0008]** VEGF-B was isolated using a yeast co-hybrid interaction trap screening technique, screening for cellular proteins which might interact with cellular retinoic acid-binding protein type I (CRABP-I). Its isolation and characteristics are described in detail in PCT/US96/02597 and in Olofsson *et al.*, Proc. Natl. Acad. Sci., 1996 93 2576-2581.

**[0009]** VEGF-C was isolated from conditioned media of PC-3 prostate adenocarcinoma cell line (CRL1435) by screening for ability of the medium to produce tyrosine phosphorylation of the endothelial cell-specific receptor tyrosine kinase ~~Flt4~~ Flt-4, using cells transfected to express ~~Flt4~~ Flt-4. VEGF-C was purified using affinity chromatography with recombinant ~~Flt4~~ Flt-4, and was cloned from a PC-3 cDNA library. Its isolation and characteristics are described in detail in Joukov *et al.*, The EMBO Journal, 1996 15 290-298.

**[0010]** VEGF2 was isolated from a highly tumorgenic, ~~oestrogen~~ estrogen-independent human breast cancer cell line. While this molecule is stated to have about 22% homology to PDGF and 30% homology to VEGF, the method of isolation of the gene encoding VEGF2 ~~is~~ was unclear, and no characterization of the biological activity ~~is~~ was disclosed.

**[0011]** Vascular endothelial growth factors appear to act by binding to receptor tyrosine kinases of the PDGF-receptor family. Five endothelial cell-specific receptor tyrosine kinases have been identified, namely Flt-1 (VEGFR-1), KDR/Flk-1 (VEGFR-2), ~~Flt4~~ Flt-4 (VEGFR-3), Tie and Tek/Tie-2. All of these have the intrinsic tyrosine kinase activity which is necessary for signal transduction. The essential, specific role in vasculogenesis and angiogenesis of Flt-1, Flk-1, Tie and

Tek/Tie-2 has been demonstrated by targeted mutations inactivating these receptors in mouse embryos. VEGFR-1 and VEGFR-2 bind VEGF with high affinity, and VEGFR-1 also binds VEGF-B and placenta growth factor (PlGF). VEGF-C has been shown to be the ligand for ~~Flt4~~ Flt-4 (VEGFR-3), and also activates VEGFR-2 (Joukov *et al.*, 1996). A ligand for Tek/Tie-2 has been described (International Patent Application No. PCT/US95/12935 (WO 96/11269) by Regeneron Pharmaceuticals, Inc.); however, the ligand for Tie has not yet been identified.

**[0012]** The receptor ~~Flt4~~ Flt-4 is expressed in venous and lymphatic endothelia in the fetus, and predominantly in lymphatic endothelia in the adult (Kaipainen *et al.*, Cancer Res., 1994 54 6571-6577; Proc. Natl. Acad. Sci. USA, 1995 92 3566-3570). It has been suggested that VEGF-C may have a primary function in lymphatic endothelium, and a secondary function in angiogenesis and permeability regulation which is shared with VEGF (Joukov *et al.*, 1996).

**[0013]** We have now isolated human cDNA encoding a novel protein of the vascular endothelial growth factor family. The novel protein, designated VEGF-D, has structural similarities to other members of this family.

## SUMMARY OF THE INVENTION

**[0014]** The invention generally provides an isolated novel growth factor which has the ability to stimulate and/or enhance proliferation or differentiation of endothelial cells, isolated DNA sequences encoding the novel growth factor, and compositions useful for diagnostic and/or therapeutic applications.

**[0015]** According to one aspect, the invention provides an isolated and purified nucleic acid molecule which encodes a novel polypeptide, designated VEGF-D, which is structurally homologous to VEGF, VEGF-B, and VEGF-C. In a preferred embodiment, the nucleic acid molecule is a cDNA which comprises the sequence set out in ~~SEQ ID NO.~~ SEQ ID NO:1, ~~SEQ ID NO.~~ SEQ ID NO:4, ~~SEQ ID NO.~~ SEQ ID NO:6 or ~~SEQ ID NO.~~ SEQ ID NO:7. This aspect of the invention also encompasses DNA molecules of sequence such that they ~~hybridise~~ hybridize under stringent conditions with DNA of ~~SEQ ID NO.~~ SEQ ID NO:1, ~~SEQ ID NO.~~ SEQ ID NO:4, ~~SEQ ID NO.~~ SEQ ID NO:6 or ~~SEQ ID NO.~~ SEQ ID NO:7. Preferably the DNA molecule able to ~~hybridise~~ hybridize under stringent conditions encodes the portion of VEGF-D from amino acid residue 93 to amino acid residue 201, and is optionally operatively linked to a DNA sequence encoding FLAG™ peptide.

**[0016]** Preferably, the cDNA comprises the sequence set out in ~~SEQ ID NO.~~ SEQ ID NO:4, ~~SEQ ID NO.~~ SEQ ID NO:6, or ~~SEQ ID NO.~~ SEQ ID NO:7, more preferably that of ~~SEQ ID NO.~~ SEQ ID NO:4.

**[0017]** According to a second aspect, the invention provides a polypeptide possessing the characteristic amino acid sequence:

Pro-Xaa-Cys-Val-Xaa-Xaa-Xaa-Arg-Cys-Xaa-Gly-Cys-Cys

(~~SEQ ID NO.~~ SEQ ID NO:2),

said polypeptide having the ability to stimulate proliferation of endothelial cells, and said polypeptide comprising a sequence of amino acids substantially corresponding to the amino acid sequence set out in ~~SEQ ID NO.~~ SEQ ID NO:3, or a fragment or ~~analogue~~ analog thereof which has the ability to stimulate one or more of

endothelial cell proliferation, differentiation, migration or survival.

**[0018]** These abilities are referred to herein as "biological activities of VEGF-D" and can readily be tested by methods known in the art. Preferably the polypeptide has the ability to stimulate endothelial cell proliferation or differentiation, including, but not limited to, proliferation or differentiation of vascular endothelial cells and/or lymphatic endothelial cells.

**[0019]** More preferably, the polypeptide has the sequence set out in ~~SEQ ID NO.~~ SEQ ID NO:5, ~~SEQ ID NO.~~ SEQ ID NO:8, or ~~SEQ ID NO.~~ SEQ ID NO:9, and most preferably has the sequence set out in ~~SEQ ID NO.~~ SEQ ID NO:5.

**[0020]** A preferred fragment of the polypeptide invention is the portion of VEGF-D from amino acid residue 93 to amino acid residue 201, and is optionally linked to FLAG™ peptide. Where the fragment is linked to FLAG™, the fragment is VEGFDΔNΔC, as hereindefined.

**[0021]** Thus, polypeptides comprising conservative substitutions, insertions, or deletions, but which still retain the biological activity of VEGF-D, are clearly to be understood to be within the scope of the invention. The person skilled in the art will be well aware of methods which can readily be used to generate such polypeptides, for example, the use of site-directed mutagenesis, or specific ~~enzymic~~ enzymatic cleavage and ligation. The skilled person will also be aware that peptidomimetic compounds or compounds in which one or more amino acid residues are replaced by a non-naturally occurring amino acid or an amino acid ~~analogue~~ analog may retain the required aspects of the biological activity of

VEGF-D. Such compounds can readily be made and tested by methods known in the art, and are also within the scope of the invention.

**[0022]** In addition, variant forms of the VEGF-D polypeptide, which result from alternative splicing, as are known to occur with VEGF, and naturally-occurring allelic variants of the nucleic acid sequence encoding VEGF-D are all encompassed within the scope of the invention. Allelic variants are well known in the art, and represent alternative forms or a nucleic acid sequence which comprise substitution, deletion or addition of one or more nucleotides, but which do not result in any substantial functional alteration of the encoded polypeptide.

**[0023]** As used herein, the term "VEGF-D" collectively refers to the polypeptides of ~~SEQ ID NO.~~ SEQ ID NO:3, ~~SEQ ID NO.~~ SEQ ID NO:5, ~~SEQ ID NO.~~ SEQ ID NO:8 and ~~SEQ ID NO.~~ SEQ ID NO:9 and fragments or ~~analogues~~ analogs thereof which have the biological activity of VEGF-D as herein defined.

**[0024]** Such variant forms of VEGF-D can be prepared by targeting non-essential regions of the VEGF-D polypeptide for modification. These non-essential regions are expected to fall outside the strongly-conserved regions indicated in the figures herein, especially Figure 2 and Figure 10. In particular, the growth factors of the PDGF family, including VEGF, are dimeric, and VEGF-B, VEGF-C, PlGF, PDGF-A and PDGF-B show complete conservation of 8 cysteine residues in the N-terminal domains, ~~ie~~ *i.e.* the PDGF-like domains (Olofsson *et al.*, 1996; Joukov *et al.*, 1996). These cysteines are thought to be involved in intra- and inter-molecular ~~disulphide~~ disulfide bonding. In addition, there are further strongly, but not

completely, conserved cysteine residues in the C-terminal domains. Loops 1, 2, and 3 of each subunit, which are formed by intra-molecular ~~disulphide~~ disulfide bonding, are involved in binding to the receptors for the PDGF/VEGF family of growth factors (Andersson *et al.*: Growth Factors, 1995 12 159-164). As shown herein, the cysteines conserved in previously known members of the VEGF family are also conserved in VEGF-D.

**[0025]** The person skilled in the art thus is well aware that these cysteine residues should be preserved in any proposed variant form, and that the active sites present in loops 1, 2, and 3 also should be preserved. However, other regions of the molecule can be expected to be of lesser importance for biological function, and therefore offer suitable targets for modification. Modified polypeptides can readily be tested for their ability to show the biological activity of VEGF-D by routine activity assay procedures such as cell proliferation tests.

**[0026]** It is contemplated that some modified VEGF-D polypeptides will have the ability to bind to endothelial cells, ~~ie~~ *i.e.* to VEGF-D receptors, but will be unable to stimulate endothelial cell proliferation, differentiation, migration, or survival. These modified polypeptides are expected to be able to act as competitive or non-competitive inhibitors of VEGF-D, and to be useful in situations where prevention or reduction of VEGF-D action is desirable. Thus, such receptor-binding but non-mitogenic, non-differentiation inducing, non-migration inducing or non-survival promoting variants of VEGF-D are also within the scope of the invention, and are referred to herein as "receptor-binding but otherwise inactive variants".

**[0027]** According to a third aspect, the invention provides a purified and isolated nucleic acid encoding a polypeptide or polypeptide fragment of the invention. The nucleic acid may be DNA, genomic DNA, cDNA, or RNA, and may be single-stranded or double stranded. The nucleic acid may be isolated from a cell or tissue source, or of recombinant or synthetic origin. Because of the degeneracy of the genetic code, the person skilled in the art will appreciate that many such coding sequences are possible, where each sequence encodes the amino acid sequence shown in ~~SEQ ID NO.~~ SEQ ID NO:3, ~~SEQ ID NO.~~ SEQ ID NO:5, ~~SEQ ID NO.~~ SEQ ID NO:8, or ~~SEQ ID NO.~~ SEQ ID NO:9, an active fragment or ~~analogue~~ analog thereof, or a receptor-binding but otherwise inactive or partially inactive variant thereof.

**[0028]** A fourth aspect of the invention provides vectors comprising the cDNA of the invention or a nucleic acid according to the third aspect of the invention, and host cells transformed or transfected with nucleic acids or vectors of the invention. These cells are particularly suitable for expression of the polypeptide of the invention, and include insect cells such as Sf9 cells, obtainable from the American Type Culture Collection (ATCC SRL-171), transformed with a baculovirus vector, and the human embryo kidney cell line 293EBNA, transfected by a suitable expression plasmid. Preferred vectors of the invention are expression vectors in which a nucleic acid according to the invention is operatively connected to one or more appropriate promoters and/or other control sequences, such that appropriate host cells transformed or transfected with the vectors are capable of expressing the polypeptide of the invention. Other preferred vectors are those suitable for transfection of mammalian cells, or for gene therapy, such as adenovirus

or retrovirus vectors or liposomes. A variety of such vectors is known in the art.

**[0029]** The invention also provides a method of making a vector capable of expressing a polypeptide encoded by a nucleic acid according to the invention, comprising the steps of operatively connecting the nucleic acid to one or more appropriate promoters and/or other control sequences, as described above.

**[0030]** The invention further provides a method of making a polypeptide according to the invention, comprising the steps of expressing a nucleic acid or vector of the invention in a host cell, and isolating the polypeptide from the host cell or from the host cell's growth medium. In one preferred embodiment of this aspect of the invention, the expression vector further comprises a sequence encoding an affinity tag, such as FLAG™ or hexahistidine, in order to facilitate purification of the polypeptide by affinity chromatography.

**[0031]** In yet a further aspect, the invention provides an antibody specifically reactive with a polypeptide of the invention. This aspect of the invention includes antibodies specific for the variant forms, fragments and ~~analogues~~ analogs of VEGF-D referred to above. Such antibodies are useful as inhibitors or agonists of VEGF-D and as diagnostic agents for detection and quantification of VEGF-D. Polyclonal or monoclonal antibodies may be used. Monoclonal and polyclonal antibodies can be raised against polypeptides of the invention using standard methods in the art. For some purposes, for example where a monoclonal antibody is to be used to inhibit effects of VEGF-D in a clinical situation, it may be desirable to use humanized or chimeric monoclonal antibodies. Methods

for producing these, including recombinant DNA methods, are also well known in the art.

**[0032]** This aspect of the invention also includes an antibody which ~~recognises~~ recognizes VEGF-D and which is suitably ~~labelled~~ labeled.

**[0033]** Polypeptides or antibodies according to the invention may be ~~labelled~~ labeled with a detectable label, and ~~utilised~~ utilized for diagnostic purposes. Similarly, the thus-~~labelled~~ labeled polypeptide of the invention may be used to identify its corresponding receptor *in situ*. The polypeptide or antibody may be covalently or non-covalently coupled to a suitable supermagnetic, paramagnetic, electron dense, ecogenic, or radioactive agent for imaging. For use in diagnostic assays, radioactive or non-radioactive labels, the latter including enzyme labels or labels of the biotin/avidin system, may be used.

**[0034]** Clinical applications of the invention include diagnostic applications, acceleration of angiogenesis in wound healing, tissue or organ transplantation, or to establish collateral circulation in tissue infarction or arterial stenosis, such as coronary artery disease, and inhibition of angiogenesis in the treatment of cancer or of diabetic retinopathy. Quantitation of VEGF-D in cancer biopsy specimens may be useful as an indicator of future metastatic risk.

**[0035]** Inasmuch as VEGF-D is highly expressed in the lung, and it also increases vascular permeability, it is relevant to a variety of lung conditions. VEGF-D assays could be used in the diagnosis of various lung disorders. VEGF-D could also be used in the treatment of lung disorders to improve blood circulation in the lung and/or gaseous exchange between the lungs and the blood stream.

Similarly, VEGF-D could be used to improve blood circulation to the heart and $O_2$ gas permeability in cases of cardiac insufficiency. In like manner, VEGF-D could be used to improve blood flow and gaseous exhange in chronic obstructive airway disease.

**[0036]** Conversely, VEGF-D antagonists (e.g., antibodies and/or inhibitors) could be used to treat ~~in~~ conditions, such as congestive heart failure, involving accumulations of fluid in, for example, the lung resulting from increases in vascular permeability, by exerting an offsetting effect on vascular permeability in order to counteract the fluid accumulation.

**[0037]** VEGF-D is also expressed in the small intestine and colon, and administrations of VEGF-D could be used to treat malabsorptive syndromes in the intestinal tract as a result of its blood circulation increasing and vascular permeabiltiy increasing activities.

**[0038]** Thus the invention provides a method of stimulation of angiogenesis and/or neovascularization in a mammal in need of such treatment, comprising the step of administering an effective dose of VEGF-D, or a fragment or ~~analogue~~ analog thereof which has the ability to stimulate endothelial cell proliferation, to the mammal.

**[0039]** Optionally VEGF-D may be administered together with, or in conjunction with, one or more of VEGF-A, VEGF-B, VEGF-C, PlGF, PDGF, FGF and/or heparin.

**[0040]** Conversely, the invention provides a method of inhibiting angiogenesis and/or neovascularization in a mammal in need of such treatment, comprising the step of administering an effective amount of an antagonist of VEGF-D to the mammal. The antagonist may be any agent

that prevents the action of VEGF-D, either by preventing the binding of VEGF-D to its corresponding receptor or the target cell, or by preventing activation of the transducer of the signal from the receptor to its cellular site of action. Suitable antagonists include, but are not limited to, antibodies directed against VEGF-D; competitive or non-competitive inhibitors of binding of VEGF-D to the VEGF-D receptor, such as the receptor-binding but non-mitogenic VEGF-D variants referred to above; and anti-sense nucleotide sequences complementary to at least a part of the DNA sequence encoding VEGF-D.

**[0041]** The invention also provides a method of detecting VEGF-D in a biological sample, comprising the step of contacting the sample with a reagent capable of binding VEGF-D, and detecting the binding. Preferably the reagent capable of binding VEGF-D is an antibody directed against VEGF-D, more preferably a monoclonal antibody. In a preferred embodiment the binding and/or extent of binding is detected by means of a detectable label; suitable labels are discussed above.

**[0042]** Where VEGF-D or an antagonist is to be used for therapeutic purposes, the dose and route of application will depend upon the condition to be treated, and will be at the discretion of the attending physician or veterinarian. Suitable routes include subcutaneous, intramuscular or intravenous injection, topical application, implants *etc.* Topical application of VEGF-D may be used in a manner analogous to VEGF.

**[0043]** According to yet a further aspect, the invention provides diagnostic/prognostic ~~means~~ device, typically in the form of test kits. For example, in one embodiment of the invention there is provided a diagnostic/prognostic test kit comprising an antibody to

VEGF-D and means for detecting, and more preferably evaluating, binding between the antibody and VEGF-D. In one preferred embodiment of the diagnostic/prognostic ~~means~~ device according to the invention, either the antibody or the VEGF-D is ~~labelled~~ labeled with a detectable label, and either the antibody or the VEGF-D is substrate-bound, such that the VEGF-D-antibody interaction can be established by determining the amount of label attached to the substrate following binding between the antibody and the VEGF-D. In a particularly preferred embodiment of the invention, the diagnostic/prognostic ~~means~~ device may be provided as a conventional ELISA kit.

**[0044]** In another alternative embodiment, the diagnostic/prognostic ~~means~~ device may comprise polymerase chain reaction means for establishing the genomic sequence structure of a VEGF-D gene of a test individual, and comparing this sequence structure with that disclosed in this application in order to detect any abnormalities, with a view to establishing whether any aberrations in VEGF-D expression are related to a given disease condition.

**[0045]** In accordance with a further aspect, the invention relates to a method of detecting aberrations in VEGF-D gene structure in a test subject which may be associated with a disease condition in said test subject. This method comprises providing a DNA sample from said test subject; contacting the DNA sample with a set of primers specific to VEGF-D DNA operatively coupled to a polymerase ~~and~~; selectively amplifying VEGF-D DNA from the sample by polymerase chain reaction~~,~~; and comparing the nucleotide sequence of the amplified VEGF-D DNA from the sample with the nucleotide sequences set forth in SEQ

ID NO:1 or SEQ ID NO:4. The invention also includes the provision of a test kit comprising a pair of primers specific to VEGF-D DNA operatively coupled to a polymerase, whereby said polymerase is enabled to selectively amplify VEGF-D DNA from a DNA sample.

**[0046]** Another aspect of the invention concerns the provision of a pharmaceutical composition comprising either VEGF-D polypeptide or a fragment or ~~analogue~~ analog thereof which promotes proliferation of endothelial cells, or an antibody thereto. Compositions which comprise VEGF-D polypeptide may optionally further comprise one or more of VEGF, VEGF-B, ~~and~~ VEGF-C, and/or heparin.

**[0047]** In another aspect, the invention relates to a protein dimer comprising VEGF-D polypeptide, particularly a ~~disulphide~~ disulfide-linked dimer. The protein dimers of the invention include both homodimers of VEGF-D polypeptide and heterodimers of VEGF-D and VEGF, VEGF-B, VEGF-C, PlGF, or PDGF.

**[0048]** According to a yet further aspect of the invention there is provided a method for isolation of VEGF-D comprising the step of exposing a cell which expresses VEGF-D to heparin to facilitate release of VEGF-D from the cell, and purifying the thus-released VEGF-D.

**[0049]** Another aspect of the invention involves providing a vector comprising an anti-sense nucleotide sequence which is complementary to at least a part of a DNA sequence which encodes VEGF-D or a fragment or ~~analogue~~ analog thereof which promotes proliferation of endothelial cells. According to a yet further aspect of the invention, such a vector comprising an anti-sense sequence may be used to inhibit, or at least mitigate,

VEGF-D expression. The use of a vector of this type to inhibit VEGF-D expression is ~~favoured~~ favored in instances where VEGF-D expression is associated with a disease, for example, where ~~tumours~~ tumors produce VEGF-D in order to provide for angiogenesis. Transformation of such ~~tumour~~ tumor cells with a vector containing an anti-sense nucleotide sequence would suppress or retard angiogenesis, and so would inhibit or retard growth of the ~~tumour~~ tumor.

**[0050]** Polynucleotides of the invention such as those described above, fragments of those polynucleotides, and variants of those polynucleotides with sufficient similarity to the non-coding strand of those polynucleotides to ~~hybridise~~ hybridize thereto under stringent conditions all are useful for identifying, purifying, and isolating polynucleotides encoding other, non-human, mammalian forms of VEGF-D. Thus, such polynucleotide fragments and variants are intended as aspects of the invention. Exemplary stringent ~~hybridisation~~ hybridization conditions are as follows: ~~hybridisation~~ hybridization at 42°C in 5X SSC, 20 mM $NaPO_4$, pH 6.8, 50% formamide; and washing at 42°C in 0.2X SSC. Those skilled in the art understand that it is desirable to vary these conditions empirically based on the length and the GC nucleotide base content of the sequences to be ~~hybridised~~ hybridized, and that formulae for determining such variation exist. See, for example, Sambrook *et al.*, "Molecular Cloning: A Laboratory Manual", Second Edition, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory (1989).

**[0051]** Moreover, purified and isolated polynucleotides encoding other, non-human, mammalian VEGF-D forms also are aspects of the invention, as are the polypeptides

encoded thereby, and antibodies that are specifically immunoreactive with the non-human VEGF-D variants. Thus, the invention includes a purified and isolated mammalian VEGF-D polypeptide, and also a purified and isolated polynucleotide encoding such a polypeptide.

**[0052]** It will be clearly understood that nucleic acids and polypeptides of the invention may be prepared by synthetic means or by recombinant means, or may be purified from natural sources.

BRIEF DESCRIPTION OF THE FIGURES

**[0053]** ~~Figure 1~~ Figures 1A-1D ~~shows~~ show a comparison between the sequences of human VEGF-D and human $VEGF_{165}$ (Figure ~~1a~~ 1A), human VEGF-B (Figure ~~1b~~ 1B), human VEGF-C (Figure ~~1c~~ 1C) and human PlGF (Figure ~~1d~~ 1D). The box indicates residues which match those in human VEGF-D exactly~~;~~.

**[0054]** Figure 2 shows sequence alignments between the sequences of human VEGF-D, human $VEGF_{165}$, human VEGF-B, human VEGF-C and human PlGF. The boxes indicate residues that match the VEGF-D sequence exactly~~; and~~.

**[0055]** Figure 3 shows the amino acid sequence of human VEGF-D (~~SEQ ID NO~~ SEQ ID NO:3), as predicted from the cDNA sequence (~~SEQ ID NO~~ SEQ ID NO:1). The boxes indicate potential sites for N-linked glycosylation.

**[0056]** Figure 4 shows the nucleotide sequence of a second cDNA sequence encoding human VEGF-D (~~SEQ ID NO~~ SEQ ID NO:4), isolated by ~~hybridisation~~ hybridization from a commercial human lung cDNA library; this cDNA contains the entire coding region for human VEGF-D~~;~~.

**[0057]** Figure 5 shows the amino acid sequence for human VEGF-D (~~SEQ ID NO~~ SEQ ID NO:5) deduced from the sequence of the cDNA of Figure 4~~;~~.

**[0058]** Figure 6 shows the nucleotide sequence of cDNA encoding mouse VEGF-D1 (~~SEQ ID NO~~ SEQ ID NO:6), isolated by ~~hybridisation~~ hybridization screening for a commercially-available mouse lung cDNA library~~;~~.

**[0059]** Figure 7 shows the nucleotide sequence of cDNA encoding mouse VEGF-D2 (~~SEQ ID NO~~ SEQ ID NO:7), isolated from the same library as in Figure 6~~;~~.

**[0060]** Figure 8 shows the deduced amino acid sequences for mouse VEGF-D1 (~~SEQ ID NO~~ SEQ ID NO:8) and VEGF-D2 (~~SEQ ID NO~~ SEQ ID NO:9)~~;~~.

**[0061]** Figure 9 shows a comparison between the deduced amino acid sequences of mouse VEGF-D1, mouse VEGF-D2, and human VEGF-D~~;~~.

**[0062]** Figure 10 shows sequence alignments between the amino acid sequences of human VEGF-D, human $VEGF_{165}$, human VEGF-B, human VEGF-C, and human PlGF~~; and~~.

**[0063]** Figure 11 shows the results of a bioassay in which conditioned medium from COS cells expressing either VEGF-A or VEGF-D was tested for ability to bind to the extracellular domain of a chimeric receptor expressed in Ba/F3 cells.

**[0064]** ~~Figure 12~~ Figures 12A-12B ~~shows~~ show the results of immunoprecipitation and Western blotting analysis of VEGF-D peptides.

(A) pEFBOSVEGFD*full*FLAG and pCDNA-1VEGF-A were transfected into COS cells and biosynthetically ~~labelled~~ labeled with $^{35}S$-cysteine/methionine for 4 hours. The supernatants from these cultures were immunoprecipitated with either M2 gel or an antiserum directed to VEGF-A

coupled to protein A. Washed beads were eluted with an equal volume of 2 x SDS-PAGE sample buffer and boiled. The samples were then resolved by 12% SDS-PAGE. Lanes marked with an asterix (*) indicate where samples were reduced with dithiothreitol and alkylated with iodoacetamide. Molecular weight markers are indicated. *f*A and *f*B indicate the 43 kD and 25 kD species immunoprecipitated by the M2 gel from the COS cells expressing pEFBOSVEGFD*full*FLAG.

(B) Western blotting analysis of purified VEGFDΔNΔC. An aliquot of material eluted from the M2 affinity column (fraction #3, VEGFDΔNΔC) was combined with 2 x SDS-PAGE sample buffer and resolved on a 15% SDS-PAGE gel. The proteins were then transferred to nitrocellulose membrane and probed with either monoclonal antibody M2 or a control isotype-matched antibody (Neg). Blots were developed using a goat anti-mouse-HRP secondary antibody and chemiluminescence (ECL , Amersham). Monomeric VEGFDΔNΔC is arrowed, as is the putative dimeric form of this peptide (VEGFDΔNΔC"). Molecular weight markers are indicated.

**[00065]** Figure 13 shows the results of analysis of VEGFDΔNΔC protein using the VEGFR2 bioassay. Recombinant VEGFDΔNΔC, and material purified by M2 affinity chromatography, was assessed using the VEGFR2 bioassay. Bioassay cells ($10^4$), washed to remove IL-3, were incubated with aliquots of conditioned medium from VEGF-D transfected COS cells, fraction #1 from the affinity column (void volume), or fraction #3 from the affinity column (containing VEGFDΔNΔC). All samples were tested at an initial concentration of 20% ~~(ie~~(i.e., 1/5) followed by doubling dilutions. Cells were allowed to incubate for 48 hours at 37°C in a humidified atmosphere of 10% $CO_2$. Cell proliferation was quantitated by the

addition of 1 μCi of $^{3}$H-thymidine and counting the amount incorporated over a period of 4 hours.

**[0066]** Figure 14 shows stimulation of tyrosine phosphorylation of the VEGFR3 receptor ( ~~Flt4~~ Flt-4) on NIH3T3 cells by culture supernatant from HF cells infected with a recombinant baculovirus vector transformed with VEGF-D.

**[0067]** Figure 15 shows stimulation of tyrosine phosphorylation of the VEGFR2 receptor (KDR) in PAE cells by culture supernatant prepared as in Figure 14.

**[0068]** Figure 16 shows the mitogenic effect of VEGFDΔNΔC on bovine aortic endothelial cells (BAEs). BAEs were treated with fraction #3 containing VEGFDΔNΔC and, as positive control, purified VEGF-A as described in the text. The result obtained using medium without added growth factor is denoted Medium Control.

## DETAILED DESCRIPTION OF THE INVENTION

**[0069]** The invention will now be described in detail by reference to the figures, and to the following non-limiting examples.

Example 1

**[0070]** It has been speculated that no further members of the VEGF family will be found, because there are no known orphan receptors in the VEGFR family. Furthermore, we are not aware of any suggestion in the prior art that other such family members would exist.

**[0071]** A computer search of nucleic acid databases was carried out incidentally to another project, using as search topics the amino acid sequences of VEGF, VEGF-B, VEGF-C, and PlGF. Several cDNA sequences were identified by this search. One of these sequences, GenBank Accession No. H24828, encoded a polypeptide which was similar in structure to the cysteine-riched C-terminal region of VEGF-C. This sequence was obtained from the database of expressed sequence tags (dbEST), and for the purposes of this specification is designated XPT. The XPT cDNA had been isolated from a human cDNA library designated "Soares Breast 3NbHBst", which was constructed using mRNA from an adult human female breast tissue. As far as can be ascertained, this sample was normal breast tissue. Sequencing of the XPT DNA was performed pursuant to the Integrated Molecular Analysis of Genome Expression Consortium (IMAGE Consortium), which solicits cDNA libraries from laboratories around the world, arrays the cDNA clones, and provides them to other ~~organisations~~ organizations for sequencing.

**[0072]** The XPT sequence shown in the database was 419 nucleotides long, and encoded an amino acid sequence similar to the C-terminal 100 amino acids of VEGF-C, ~~ie.~~ *i.e.*, approximately residues 250 to 350, using the numbering system of Joukov *et al.* (1996). Similarly, cysteine-rich regions are found in other proteins, which are entirely unrelated in function to the VEGF family,

for example, the secreted silk-like protein sp185 synthesized in the salivary glands of the midge *Chironomus tentans*. This protein is encoded by the gene BR3, located in a Balbiani ring, a tissue specific chromosome "puff" found on polytene chromosomes in the midge salivary gland (Dignam and Case: Gene, 1990 88 133-140; Paulsson *et al.*, J. Mol. Biol., 1990 211 331-349). It is stated in Joukov *et al.* (1996) that the sp185-like structural motif in VEGF-C may fold into an independent domain, which is thought to be at least partially cleaved off after biosynthesis, and that there is at least one cysteine motif of the sp185 type in the C-terminal region of VEGF.

**[0073]** Figure 3 of Joukov *et al.* shows that the last two-thirds of the C-terminal cysteine-rich region of VEGF-C do not align with VEGF or PlGF, and in fact could be considered a C-terminal extension of VEGF-C which is not present in VEGF or PlGF. The sequence encoded by XPT is similar to this extension. As the XPT cDNA was truncated at its 5' end, it was not possible to deduce or predict any amino acid sequence for regions N-terminal to the cysteine-rich domain. Thus the portion of VEGF-C which is similar to the XPT-derived sequence does not extend to regions of VEGF-C which are conserved among other members of the VEGF family.

**[0074]** As described above, it was not possible to predict whether the N-terminal region of the polypeptide encoded by a full-length XPT nucleic acid (as distinct from the truncated XPT cDNA reported in dbEST) would show any further homology to any member of the VEGF family, in particular VEGF-C, which has a further N-terminal 250 amino acids. For example, the naturally-occurring protein encoded by a full-length XPT nucleic acid could

have been the human ~~homologue~~ homolog of the midge salivary gland protein. Alternatively, the type of cysteine-rich motif encoded by truncated XPT cDNA could be widely distributed among proteins, as are many structural domains. For example, clusters of cysteine residues may be involved in metal binding, formation of intramolecular ~~disulphide~~ disulfide bonds to promote accurate protein folding, or formation of intermolecular ~~disulphide~~ disulfide bonds for assembly of protein subunits into complexes (Dignam and Chase, 1990). In order to determine whether the truncated XPT cDNA was derived from sequences encoding a VEGF-related molecule, it was necessary to isolate a much longer cDNA.

Example 2 Cloning of cDNA Encoding VEGF-D

**[0075]** A sample of the XPT cDNA reported in dbEST was obtained from the American Type Culture Collection, which is a registered supplier of cDNA clones obtained by the IMAGE Consortium. The identity of the XPT cDNA was confirmed by nucleotide sequencing, using the dideoxy chain termination method (Sanger *et al.*, Proc. Natl. Acad. Sci. USA, 1977 74 5463-5467).

**[0076]** The XPT cDNA was used as a ~~hybridisation~~ hybridization probe to screen a human breast cDNA library, which was obtained commercially from Clontech. One positive clone was isolated, and this clone was then sequenced on both strands. The nucleotide sequence was compiled, and an open reading frame was identified. The nucleic acid sequence is set out in ~~SEQ ID NO.~~ SEQ ID NO:1. The polypeptide encoded by this sequence was designated VEGF-D, and its deduced amino acid sequence, designated ~~SEQ ID NO.~~ SEQ ID NO:3, is set out in Figure 3. In Figure 3, putative sites of N-linked glycosylation,

with the consensus sequence N-X-S/T in which X is any amino acid, are indicated by the boxes.

Example 3 Characteristics of VEGF-D

**[0077]** The amino acid sequence of VEGF-D was compared with those of human VEGF-$A_{165}$, VEGF-B, VEGF-C, and PlGF. These comparisons are set out in Figures ~~1a to d~~ 1A to D, respectively. The degree of sequence homology was calculated, and if gaps in sequence introduced for the purposes of alignment are not considered in the calculation, VEGF-D is 31% identical to VEGF, 48% identical to VEGF-C, 28% identical to VEGF-B, and 32% identical to PlGF. Thus, the most closely-related protein identified was VEGF-C.

**[0078]** Computer searches of the GenBank, EMBL and SwissProt nucleic acid databases did not reveal any protein sequences identical to VEGF-D. As expected from the sequence alignment referred to above, the most closely related protein found in these databases was VEGF-C. Searches of dbEST were also performed, but did not reveal any sequences encompassing the entire coding region of VEGF-D. The sequence of VEGF-D is unrelated to that of Tie-2 ligand 1 as disclosed in WO 96/11269.

**[0079]** It is important to bear in mind that the only homologies detected were at the level of the amino acid sequence. Thus, it would not have been possible to isolate the cDNA or gDNA encoding VEGF-D by methods such as low-stringency hybridization with a nucleic acid sequence encoding another member of the VEGF family.

**[0080]** VEGF-D appears to be most closely related to VEGF-C of all the members of the VEGF family. Because the VEGF-D amino acid sequence includes the cysteine-rich sp185-like motif which is found in VEGF-C, the

polypeptide of the invention may play an important functional role in lymphatic endothelia. While we do not wish to be bound by any proposed mechanism, it is thought that VEGF-C and VEGF-D may constitute a silk-like matrix over which endothelial cells can grow. Lymphatic vessels have no basement membrane, so the silk-like matrix can form a basement membrane-like material. This may be important in promoting cell growth and/or in cell differentiation, and may be relevant to cancer, especially metastasis, drug therapy, cancer prognosis, *etc.*

Example 4 Biological Characteristics of VEGF-D

**[0081]** The cDNA sequence of VEGF-D was used to predict the deduced amino acid sequence of VEGF-D, the biochemical characteristics of the encoded polypeptide, including the numbers of strongly basic, strongly acidic, hydrophobic and polar amino acids, the molecular weight, the isoelectric point, the charge at pH 7, and the compositional analysis of the whole protein. This analysis was performed using the Protean protein analysis program, Version 1.20 (DATASTAR). These results are ~~summarised~~ summarized in Tables 1 and 2 below. Table 1 also shows the codon usage.

Table 1
Translated DNA Sequence of VEGF-D contig x(1,978)
With Standard Genetic Code

Molecular Weight 37056.60 Daltons
425 Amino Acids
46 Strong Basic(+) Amino Acids (K,R)
41 Strong Acidic(-) Amino Acids (D,E)
79 hydrophobic Amino Acids (A,I,L,F,W,V)

108 Polar Amino Acids (N,C,Q,S,T,Y)

7.792 Isoelectric Point
6.371 Charge at pH 7.0

Total number of bases translated is 978
% A = 28.73 [281]
% G = 23.11 [226]
% T = 23.21 [227]
% C = 24.95 [244]
% Ambiguous = 0.00 [0]
% A+T = 51.94 [508]
% C+G = 48.06 [470]

Davis, Botstein, Roth Melting Temp °C. 84.09
Wallace Temp °C. 3384.00

Table 1 (cont.)

| Codon usage: | | | | |
|---|---|---|---|---|
| ccg ( ) | 0 # ugc Cys(C) | 14 # cuc Leu(L) | 6 # ucg Ser(S) | |
| uaa ( ) | 0 # ugu Cys(C) | 14 # cuc Leu(L) | 6 # ucg Ser(S) | |
| uag ( ) | 0 # --- Cys(C) | 30 # cuu Leu(L) | 2 # --- Ser(S) | 3 |
| --- ( ) | 0 # caa Gln(Q) | 1 # uua Leu(L) | 1 # uga Ter(.) | |
| gca Ala(A) | 5 # cag Gln(Q) | 11 # uug Leu(L) | 5 # --- Ter(.) | |
| gcc Ala(A) | 4 # --- Gln(Q) | 12 # --- Leu(L) | 23 # aca Thr(T) | |
| gca Ala(A) | 1 # gaa Glu(E) | 16 # aaa Lys(K) | 13 # acc Thr(T) | |
| gcu Ala(A) | 5 # gag Glu(E) | 12 # aag Lys(K) | 10 # acg Thr(T) | |
| --- Ala(A) | 15 # --- Glu(E) | 28 # --- Lys(K) | 23 # acu Thr(T) | |
| aga Arg(R) | 7 # gga Gly(G) | 1 # aug Met(M) | 6 # --- Thr(T) | 2 |
| agg Arg(R) | 5 # ggc Gly(G) | 2 # --- Met(M) | 6 # ugg Trp(W) | |
| cga Arg(R) | 5 # ggg Gly(G) | 3 # uuc Phe(F) | 4 # --- Trp(W) | |
| cgc Arg(R) | 4 # ggu Gly(G) | 2 # uuu Phe(F) | 8 # uac Tyr(Y) | |
| cgg Arg(R) | 1 # --- Gly(G) | 8 # --- Phe(F) | 12 # uau Tyr(Y) | |
| cgu Arg(R) | 1 # cac His(H) | 7 # cca Pro(P) | 9 # --- Tyr(Y) | |
| --- Arg(R) | 23 # cau His(H) | 7 # ccc Pro(P) | 6 # gua Val(V) | |
| aac Asn(N) | 5 # --- His(H) | 14 # ccu Pro(P) | 8 # guc Val(V) | |
| aau Asn(N) | 4 # aua Ile(I) | 2 # --- Pro(P) | 23 # gug Val(V) | |
| --- Asn(N) | 9 # auc Ile(I) | 6 # agc Ser(S) | 6 # guu Val(V) | |
| gac Asp(D) | 8 # auu Ile(I) | 5 # agu Ser(S) | 8 # --- Val(V) | 1 |
| qau Asp(D) | 5 # --- Ile(I) | 13 # uca Ser(S) | 5 # nnn ???(X) | |
| gau Asp(D) | 5 # --- Ile(I) | 13 # uca Ser(S) | 5 # nnn ???(X) | |
| --- Asp(D) | 13 # cua Leu(L) | 5 # ucc Ser(S) | 7 # TOTAL | 32 |

Contig 2:

Contig Length: 2379 bases

Average Length/Sequence: 354 bases

Total Sequence Length: 4969 bases

Table 2

Predicted Structural Class of the Whole Protein:

Deléage & Roux Modification of Nishikawa & Ooi 1987

| **Analysis** | **Whole Protein** |
|---|---|
| Molecular Weight | 37056.60 m.w. |
| Length | 325 |
| 1 microgram = | 26.986 pMoles |
| Molar Extinction coefficient | 30200±5% |
| 1 A(280) = | 1.23 mg/ml |
| Isoelectric Point | 7.79 |
| Charge at pH 7 | 6.37 |

Table 2 (cont.)

Whole Protein Composition ~~Anayalsis~~ Analysis

| Amino Acid(s) | Number count | % by weight | % by frequency |
|---|---|---|---|
| Charged (RKHYCDE) | 134 | 46.30 | 41.23 |
| Acidic (DE) | 41 | 13.79 | 12.62 |
| Basic (KR) | 46 | 17.65 | 14.15 |
| Polar (NCQSTY) | 108 | 30.08 | 33.23 |
| Hydrophobic (AILFWV) | 79 | 23.86 | 24.31 |
| A Ala | 15 | 2.88 | 4.62 |
| C Cys | 30 | 8.35 | 9.23 |
| D Asp | 13 | 4.04 | 4.00 |
| E Glu | 28 | 9.75 | 8.62 |
| F Phe | 12 | 4.77 | 3.69 |
| G Gly | 8 | 1.23 | 2.46 |
| H His | 14 | 5.18 | 4.31 |
| I Ile | 13 | 3.97 | 4.00 |
| K Lys | 23 | 7.96 | 7.08 |
| L Leu | 23 | 7.03 | 7.08 |
| M Met | 6 | 2.12 | 1.85 |
| N Asn | 9 | 2.77 | 2.77 |
| P Pro | 23 | 6.08 | 7.08 |
| Q Gln | 12 | 4.15 | 3.69 |
| R Arg | 23 | 9.69 | 7.08 |
| S Ser | 33 | 7.76 | 10.15 |
| T Thr | 21 | 5.73 | 6.46 |
| V Val | 12 | 3.21 | 3.69 |
| ~~W Trp~~ | ~~4~~ | ~~2.01~~ | ~~1.23~~ |
| Y Trp | 3 | 1.32 | 0.92 |
| B Asx | 0 | 0.00 | 0.00 |
| Z Glx | 0 | 0.00 | 0.00 |
| X Xxx | 0 | 0.00 | 0.00 |
| . Ter | 0 | 0.00 | 0.00 |

**[0082]** This analysis predicts a molecular weight for the unprocessed VEGF-D monomer of 37 kilodaltons (kD), compared to the experimentally determined values (for the fully processes peptides) of 20 to 27 kD for VEGF-A monomers, 21 kD for the VEGF-B monomer and 23 kD for the VEGF-C monomer.

Example 5

**[0083]** The original isolation of a cDNA for VEGF-D, described in Example 2 involved ~~hybridisation~~ hybridization screening of a human breast cDNA library. As only one cDNA clone for VEGF-D was thus isolated, it was not possible to confirm the structure of the cDNA by comparison with other independently isolated VEGF-D cDNAs. The work described in this example, which involved isolation of additional human VEGF-D cDNA clones, was carried out in order to confirm the structure of human VEGF-D cDNA. In addition, mouse VEGF-D cDNA clones were isolated.

**[0084]** Two cDNA libraries which had been obtained commercially from Stratagene, one for human lung and one for mouse lung (catalogue numbers 937210 and 936307, respectively) were used for ~~hybridisation~~ hybridization screening with a VEGF-D cDNA probe. The probe, which spanned from nucleotides 1817 to 2495 of the cDNA for human VEGF-D described in Example 2, was generated by polymerase chain reaction (PCR) using a plasmid containing the VEGF-D cDNA as template and the following two oligonucleotides:

5'-GGGCTGCTTCTAGTTTGGAG (~~SEQ ID NO.~~ SEQ ID NO:10), and

5'-CACTCGCAACGATCTTCGTC (~~SEQ ID NO.~~ SEQ ID NO:11).

**[0085]** Approximately two million recombinant bacteriophage were screened with this probe from each of

the two cDNA libraries. Nine human and six mouse cDNA clones for VEGF-D were subsequently isolated.

[00086] Two of the nine human cDNA clones for VEGF-D were sequenced completely using the dideoxy chain termination method (Sanger *et al.*, Proc. Natl. Acad. Sci. USA, 1977 74 5463-5467). The two cDNAs contained the entire coding region for human VEGF-D, and were identical except that one of the clones was five nucleotides longer than the other at the 5'-terminus. The nucleotide sequence of the shorter cDNA is shown in Figure 4, and is designated ~~SEQ ID NO.~~ SEQ ID NO:4. The amino acid sequence for human VEGF-D (hVEGF-D) deduced from this cDNA was 354 residues long, and is shown in Figure 5; this is designated ~~SEQ ID NO.~~ SEQ ID NO:5. The sequences of the 5' regions of five of the other human VEGF-D cDNA clones were also determined. For each clone, the sequence that was characterized contained more than 100 nucleotides of DNA immediately downstream from the translation start site of the coding region. In all cases, the sequences of these regions were identical to corresponding regions of the human VEGF-D cDNA shown in Figure 4.

[00087] All six mouse cDNA clones for VEGF-D were sequenced completely. Only two of the clones contained an entire coding region for VEGF-D; the other clones were truncated. The nucleotide sequences of the two clones with an entire coding region are different, and encode amino acid sequences of different sizes. The longer amino acid sequence is designated mVEGF-D1, and the shorter sequence is designated mVEGF-D2. The nucleotide sequences of the cDNAs encoding mVEGF-D1 and mVEGF-D2 are shown in Figures 6 and 7 respectively. The deduced amino acid sequences for mVEGF-D1 and mVEGF-D2 are shown in Figure 8. These sequences are respectively designated SEQ ID ~~NOS. 6, 7, 8 and~~ NO: 6, SEQ ID NO: 7, SEQ ID NO: 8 and SEQ ID NO: 9. The differences between the amino acid sequences are:

i) an insertion of five amino acids (DFSFE) after residue 30 in mVEGF-D1 in comparison to mVEGF-D2;

ii) complete divergence of the C-terminal ends after residue 317 in mVEGF-D1 and residue 312 in mVEGF-D2, which results in mVEGF-D1 being considerably longer.

**[0088]** Three of the four truncated cDNAs for mouse VEGF-D encoded the C-terminal region, but not the N-terminal 50 amino acids. All three of these cDNAs encoded a C-terminal end for VEGF-D which is identical to that for mVEGF-D2. The other truncated cDNA encoded only the N-terminal half of VEGF-D. The amino acid sequence deduced from this cDNA contained the five amino acids DFSFE immediately after residue 30 found in mVEGF-D1, but not in mVEGF-D2.

**[0089]** As described above, the entire sequence of the human VEGF-D cDNA clone reported in this example has been validated by comparison with that for a second human clone. In addition, the sequence of the 5' end of the coding region was found to be identical in five other human VEGF-D cDNA clones. In contrast, the sequence reported in Example 2 contained most of the coding region for VEGF-D, but was incorrect near the 5'-end of this region. This was probably because the VEGF-D cDNA was truncated near the 5'-end of the coding region and at that point had been ligated with another unidentified cDNA, and consequently the first 30 codons of the true coding sequence for VEGF-D had been deleted and replaced with a methionine residue. This methionine residue was defined as the N-terminal amino acid of the VEGF-D sequence presented in Example 2.

**[0090]** The N-terminal regions of the deduced amino acid sequences of mouse VEGF-D1 and VEGF-D2 are very similar to that deduced for human VEGF-D (see Figure 9). This also indicates that the correct deduced amino acid sequence for human VEGF-D is reported in this example. The N-terminal

25 amino acids of human VEGF-D form an extremely hydrophobic region, which is consistent with the notion that part of this region may be a signal sequence for protein secretion. Figure 10 shows the alignment of the human VEGF-D sequence with the sequences of other members of the VEGF family of growth factors, namely human $VEGF_{165}$ ($hVEGF_{165}$), human VEGF-B (hVEGF-B), human VEGF-C (hVEGF-C), and human Placental Growth Factor (hPlGF). When gaps in the alignments are ignored for the purposes of calculation, human VEGF-D is found to be 31% identical in amino acid sequence to human $VEGF_{165}$, 28% identical to human VEGF-B, 48% identical to VEGF-C and 32% identical to human PlGF. Clearly, VEGF-C is the member of this family which is most closely related to VEGF-D.

**[0091]** The differences in sequence for mouse VEGF-D1 and VEGF-D2 most probably arise from differential mRNA splicing. The C-terminal 41 amino acid residues of VEGF-D1 are deleted in VEGF-D2, and are replaced with 9 residues which are not closely related to the VEGF-D1 sequence. Therefore, 4 cysteine residues present near the C-terminus of VEGF-D1 are deleted in VEGF-D2. This change may alter the tertiary or quaternary structures of the protein, or may affect the ~~localisation~~ localization of the protein in the cell or the extracellular environment. The C-terminal end of human VEGF-D resembles that of mouse VEGF-D1, not mouse VEGF-D2. The small 5 amino acid insertion after residue 30 in mouse VEGF-D1, which is not present in either mouse VEGF-D2 or human VEGF-D, may influence proteolytic processing of the protein.

**[0092]** VEGF-D is highly conserved between mouse and man. Eighty-five percent of the amino acid residues of human VEGF-D are identical in mouse VEGF-D1. This is likely to reflect conservation of protein function. Putative functions for VEGF-D have been proposed herein.

Although we have not found alternative forms of human VEGF-D cDNA, it is possible that the RNA splice variation which gives rise to numerous forms of mRNA for mouse VEGF-D may also occur in human tissues.

Example 6 Expression of VEGF-D in COS Cells

**[0093]** A fragment of the human cDNA for VEGF-D, spanning from nucleotide 1 to 1520 of the sequence shown in Figure 4 and containing the entire coding region, was inserted into the mammalian expression vector pCDNA1-amp. The vector was used to transiently transfect COS cells by the DEAE-Dextran method as described previously (Aruffo and Seed, 1987) and the resulting conditioned cell culture media, collected after 7 days of incubation, were concentrated using Amicon concentrators (Centricon 10 with a 10,000 molecular weight cut off) according to the manufacturer. The plasmids used for transfections were the expression construct for human VEGF-D and, as positive control, a construct made by insertion of mouse VEGF-A cDNA into pCDNA1-amp. The conditioned media were tested in two different bioassays, as described below, and the results demonstrate that the COS cells did, in fact, express and secrete biologically-active VEGF-D.

Example 7 Bioassay for Capacity of VEGF-D to Bind to VEGF Receptor-2

**[0094]** As shown in Example 5, VEGF-D is closely related in primary structure to other members of the VEGF family. Most members of this protein family are mitogenic and/or chemotactic for endothelial cells (Keck *et al.*, 1989; Leung *et al.*, 1989; Joukov, *et al.*, 1996; Olofsson *et al* ~~1996 ).~~ ., 1996). In addition, VEGF-A (previously known as VEGF), the first member of the VEGF family to be described in the literature, is a potent inducer of vascular permeability (Keck *et al.*, 1989). As protein structure is an important determinant of protein function, it seemed likely that VEGF-D might also be mitogenic for endothelial cells or induce vascular permeability. Therefore human VEGF-D was tested in a bioassay for its capacity to bind to VEGF receptor-2 (VEGFR2; also known as Flk-1), an endothelial cell-specific receptor which, when activated by VEGF-A, is thought to give rise to a mitogenic signal (Strawn *et al.*, 1996).

**[0095]** A bioassay for detection of growth factors which bind to VEGFR2 has been developed in the factor-dependent cell line Ba/F3, and is described in our earlier patent application, No. PCT/US95/16755. These cells grow in the presence of interleukin-3 (IL-3); however, removal of this factor results in cell death within 48 hours. If another receptor capable of delivering a growth stimulus is transfected into the Ba/F3 cells, the cells can be rescued by the specific growth factor which activates that receptor when the cells are grown in medium lacking IL-3. In the specific case of receptor-type tyrosine kinases ~~(eg.~~(e.g., VEGFR2), chimeric receptors containing the extracellular domain of the receptor tyrosine kinase and the transmembrane and cytoplasmic domains of the erythropoietin receptor (EpoR) can be ~~utilised~~ utilized. In this case stimulation with the ligand ~~(eg.~~(e.g., VEGF),

which binds to the extracellular domain of the chimeric receptor, results in signalling via the EpoR cytoplasmic domain and subsequent rescue of the cell line in growth medium lacking IL-3. The construction of the chimeric receptor used in this study, consisting of the mouse VEGFR2 extracellular domain and the mouse EpoR transmembrane and cytoplasmic domains, and the bioassay itself, are described below.

*Plasmid Construction*

i) Construction of a plasmid for generating chimeric VEGFR2 receptors

**[0096]** To obtain a plasmid construct with which DNA encoding the extracellular domain of mouse VEGFR2 could easily be ligated with DNA encoding other protein domains, site-directed mutagenesis was used to generate a BglII restriction enzyme site at the position of mouse VEGFR2 cDNA which encoded the junction of the extracellular domain and the transmembrane domain. The full-length clone of the mouse VEGFR2 cDNA described by Oelrichs *et al.* (1993) was subcloned into the mammalian expression vector pCDNA1-amp, using the BstXI restriction enzyme site. Single stranded UTP+ DNA was generated using the M13 origin of replication, and this was used as a template to generate mouse VEGFR2 cDNA containing the BglII site at the desired position. The plasmid containing the altered VEGFR2 cDNA was designated pVEGFR2Bgl. DNA fragments encoding the transmembrane and cytoplasmic domains of any receptor can be inserted at the BglII site of pVEGFR2Bgl in order to generate chimeric VEGFR2 receptors.

ii) Construction of VEGFR2/EpoR chimeric receptor

**[0097]** The mouse EpoR cDNA was subcloned into the expression vector pCDNA1-amp, and single stranded DNA was generated as a template for mutagenesis. A BglII

restriction enzyme site was inserted into the EpoR cDNA at the position encoding the junction of the transmembrane and extracellular domains of the EpoR to allow direct ligation of this DNA fragment to the modified cDNA encoding the extracellular domain of VEGFR2 in pVEGFR2Bgl. In addition, a BglII site in the cytoplasmic domain of the EpoR was removed by a silent single nucleotide substitution. The DNA fragment encoding the transmembrane and cytoplasmic domains of EpoR was then used to replace the portion of pVEGFR2Bgl encoding the transmembrane and cytoplasmic domains of VEGFR2. Thus a single reading frame was generated which encoded the chimeric receptor consisting of the VEGFR2 extracellular domain and the EpoR transmembrane and cytoplasmic domains.

**[0098]** The DNA fragment encoding the chimeric receptor was subcloned into the expression vector pBOS, and co-transfected into the Ba/F3 cell line with plasmid pgk-neo at a ratio of 1:20. Cells expressing the VEGFR2-EpoR protein were selected by flow cytometry analysis using a monoclonal antibody to the VEGFR2 extracellular domain (MAb 4H3). This monoclonal antibody is described in Australian Patent Application No. PM 3794, filed 10 February 1994. Cell lines expressing higher levels of VEGFR2-EpoR were selected by growing the cells in 5 µg/ml MAb 4H3 or 25 ng/ml of recombinant VEGF. A cell line expressing high levels of VEGFR2-EpoR, designated Ba/F3-NYK-EpoR, was used for the bioassay.

*The Bioassay*

**[00099]** The Ba/F3-NYK-EpoR cells described above were washed three times in PBS to remove all IL-3 and resuspended at a concentration of 1000 cells per 13.5 µl of culture medium and 13.5 µl was aliquoted per well of a 60-well Terasaki plate. Conditioned media from transfected COS cells were then diluted into the cell culture medium. Cells expressing a chimeric receptor consisting of the extracellular domain of the endothelial cell receptor Tie2 and the transmembrane and cytoplasmic domains of EpoR were used as a non-responding control cell line. Cells were incubated for 48-96 hours, during which the cells incubated in cell culture medium alone had died and the relative survival/proliferation seen in the other wells ~~(ie.~~(i.e., in the presence of COS cell-conditioned media) was scored by counting the viable cells present per well.

**[00100]** The conditioned medium from COS cells which had been transiently transfected with expression plasmids was concentrated 30-fold and used in the VEGFR2 bioassay. Concentrated conditioned medium from COS cells transfected with pCDNA1-amp was used as negative control.

**[00101]** The results are shown in Figure 11, with the percentage of 30-fold concentrated COS cell-conditioned medium in the incubation medium (vol/vol) plotted versus the number of viable cells in the well after 48 hours of incubation. Clearly, the conditioned medium containing either VEGF-A or VEGF-D was capable of promoting cell survival in this assay, indicating that both proteins can bind to and activate VEGFR2.

Example 8 Vascular Permeability Assay

[00102] Human VEGF-D, prepared as in Example 6 and concentrated 30-fold, was tested in the Miles vascular permeability assay (Miles and Miles, 1952) performed in anaesthetized guinea pigs (albino/white, 300-400 g). Concentrated conditioned medium for COS cells transfected with pCDNA1-amp was again used as a negative control. Guinea pigs were ~~anaesthetised~~ anaesthetized with chloral-hydrate (3.6 g/100 ml; 0.1 ml per 10 g of body weight). The backs of the animals were then carefully shaved with clippers. Animals were given an intracardiac injection of Evans Blue dye (0.5% in MT PBS, 0.5 ml) using a 23G needle, and were then injected intra-dermally with 100-150 µl of concentrated COS cell-conditioned medium. After 15-20 min the animals were sacrificed and the layer of skin on the back excised to expose the underlying blood vessels. For quantitation, the area of each injection was excised and heated to 45°C in 2-5 ml of formamide. The resulting supernatants, containing extravasated dye, were then examined spectrophotometrically at 620 nm.

[00103] For animal 1, the absorbance at 620 nm arising from injection of 30-fold concentrated VEGF-A conditioned medium was 0.178, that for the 30-fold concentrated VEGF-D conditioned medium was 0.114, and that for 30-fold concentrated medium from cells transfected with pCDNA1-amp was 0.004. For animal 2, the 30-fold concentrated media were diluted 4-fold in cell culture medium before intra-dermal injection. The absorbance at 620 nm for the VEGF-A conditioned sample was 0.141, that for the VEGF-D conditioned sample was 0.116, and that for a sample matched for serum content as negative control was 0.017. The enhanced extravasation of dye observed for both animals in the presence of VEGF-A or VEGF-D demonstrated that both of these proteins strongly induced vascular permeability.

[00104] The data described here indicate that VEGF-D is a secreted protein which, like VEGF-A, binds to and activates VEGFR2 and can induce vascular permeability.

Example 9 Bioactivities of Internal VEGF-D Polypeptides

[00105] The deduced amino acid sequence for VEGF-D includes a central region which is similar in sequence to all other members of the VEGF family (approximately residues 101 to 196 of the human VEGF-D amino acid sequence as shown in the alignment in Figure 10). Therefore, it was thought that the bioactive portion of VEGF-D might reside in the conserved region. In order to test this hypothesis, the biosynthesis of VEGF-D was studied, and the conserved region of human VEGF-D was expressed in mammalian cells, purified, and tested in bioassays as described below.

*Plasmid construction*

[00106] A DNA fragment encoding the portion of human VEGF-D from residue 93 to 201, ~~*ie.*~~ *i.e.,* with N- and C-terminal regions removed, was amplified by polymerase chain reaction with *Pfu* DNA polymerase, using as template a plasmid comprising full-length human VEGF-D cDNA. The amplified DNA fragment, the sequence of which was confirmed by nucleotide sequencing, was then inserted into the expression vector pEFBOSSFLAG to give rise to a plasmid designated pEFBOSVEGFDΔNΔC. The pEFBOSSFLAG vector contains DNA encoding the signal sequence for protein secretion from the interleukin-3 (IL-3) gene and the FLAG™ octapeptide. The FLAG™ octapeptide can be recognized by commercially available antibodies such as the M2 monoclonal antibody (IBI/Kodak). The VEGF-D PCR fragment was inserted into the vector such that the IL-3 signal sequence was immediately upstream from the FLAG™ sequence, which was in turn immediately upstream from the

VEGF-D sequence. All three sequences were in the same reading frame, so that translation of mRNA resulting from transfection of pEFBOSVEGFDΔNΔC into mammalian cells would give rise to a protein which would have the IL-3 signal sequence at its N-terminus, followed by the FLAG™ octapeptide and the VEGF-D sequence. Cleavage of the signal sequence and subsequent secretion of the protein from the cell would give rise to a VEGF-D polypeptide which is tagged with the FLAG™ octapeptide adjacent to the N-terminus. This protein was designated VEGFDΔNΔC.

**[00107]** In addition, a second plasmid was constructed, designated pEFBOSVEGFD*full*FLAG, in which the full-length coding sequence of human VEGF-D was inserted into pEFBOSIFLAG such that the sequence for the FLAG™ octapeptide was immediately downstream from, and in the same reading frame as, the coding sequence of VEGF-D. The plasmid pEFBOSIFLAG lacks the IL-3 signal sequence, so secretion of the VEGF-D/FLAG fusion protein was driven by the signal sequence of VEGF-D. pEFBOSVEGFD*full*FLAG was designed to drive expression in mammalian cells of full-length VEGF-D which was C-terminally tagged with the FLAG™ octapeptide. This protein is designated VEGFD*full*FLAG, and is useful for the study of VEGF-D biosynthesis.

*Analysis of the Post-Translational Processing of VEGF-D*

**[00108]** To examine whether the VEGF-D polypeptide is processed to give a mature and fully active protein, pEFBOSVEGFD*full*FLAG was transiently transfected into COS cells (Aruffo and Seed, 1987). Expression in COS cells followed by biosynthetic labeling with $^{35}$S-methionine/cysteine and immunoprecipitation with M2 gel has demonstrated species of approximately 43 kD (*f*A) and 25 kD(*f*B) (Figure 12A). These bands are consistent with the notion that VEGF-D is cleaved to give a C-terminal

fragment (FLAG™ tagged) and an internal peptide (corresponding approximately to the VEGFDΔNΔC protein). Reduction of the immunoprecipitates (M2*) gives some reduction of the *f*A band, indicating the potential for disulphide linkage between the two fragments.

*Expression and purification of internal VEGF-D polypeptide*

**[00109]** Plasmid pEFBOSVEGFDΔNΔC was used to transiently transfect COS cells by the DEAE-Dextran method as described previously (Aruffo and Seed, 1987). The resulting conditioned cell culture medium (approximately 150 ml), collected after 7 days of incubation, was subjected to affinity chromatography using a resin to which the M2 monoclonal antibody had been coupled. In brief, the medium was run batch-wise over a 1 ml M2 antibody column for approximately 4 hours at ~~4 C~~ 4°C. The column was then washed extensively with 10 mM Tris-HCl, pH 8.0, 150 mM NaCl before elution with free FLAG™ peptide at 25 µg/ml ~~g/ml~~ in the same buffer. The resulting material was used for the bioassays described below.

**[00110]** In order to detect the purified VEGFDΔNΔC, fractions eluted from the M2 affinity column were subjected to Western blot analysis. Aliquots of the column fractions were combined with 2 x SDS-PAGE sample buffer, boiled, and loaded onto a 15% SDS polyacrylamide gel. The resolved fractions were transferred to nitrocellulose membrane and non-specific binding sites blocked by incubation in Tris/NaCl/Tween 20 (TST) and 10% skim milk powder (BLOTTO). Membranes were then incubated with monoclonal antibody M2 or control antibody at 3 µg/ml for 2 h at room temperature, followed by extensive washing in TST. Membranes were then incubated with a secondary goat anti-mouse HRP-conjugated antiserum for 1 h at room temperature, followed by washing in TST buffer. Detection

of the protein species was achieved using a chemiluminescent reagent (ECL, Amersham) (Figure 12B).

**[00111]** Under non-reducing conditions a species of molecular weight approximately 23 kD (VEGFDΔNΔC) was detected by the M2 antibody. This is consistent with the predicted molecular weight for this internal fragment (12,800) plus N-linked glycosylation; VEGFDΔNΔC contains two potential N-linked glycosylation sites. A species of approximately 40 kD was also detected, and may represent a non-covalent dimer of the 23 kD protein (VEGFDΔNΔC).

*Bioassays*

**[00112]** The bioassay for the capacity of polypeptides to bind to VEGF receptor-2 is described in detail in Example 7. Aliquots of fractions eluted from the M2 affinity column, containing the VEGFDΔNΔC protein, were diluted in medium and tested in the VEGFR2 bioassay as previously described. Fraction #3 from the affinity column, which was shown to contain the purified VEGFDΔNΔC protein (Figure 12B), demonstrated a clear ability to induce proliferation of the bioassay cell line to a dilution of 1/100 of the purified fraction (Figure 13). In comparison, the void volume of the affinity column (fraction #1) showed no activity, whereas the original VEGFDΔNΔC conditioned medium gave only weak activity.

**[00113]** The vascular permeability assay (Miles and Miles, 1952) is described in brief in ~~example~~ Example 8. Aliquots of purified VEGFDΔNΔC, and samples of the void volume from the M2 affinity column (negative control) were combined with medium and injected intradermally into the skin of guinea pigs. The regions of skin at the sites of injections were excised, and extravasated dye was eluted. The absorbance of the extravasated dye at 620 nm arising from injection of purified VEGFDΔNΔC was 0.131 ± ± 0.009. In comparison, the value for absorbance arising from

injection of a sample of the void volume was 0.092 ± ± 0.020. Therefore, VEGFDΔNΔC induced vascular permeability, but the effect was only marginal.

**[00114]** Due to its ability to bind to VEGFR2, and its lower induction of vascular permeability compared to full length VEGF-D, VEGF-DΔNΔC may be said to relatively decrease the induction of vascular permeability by VEGF-D through competitive inhibition. In this sense, the VEGF-DΔNΔC fragment may be thought of as an antagonist for VEGF-D as regards the induction of vascular permeability.

*Summary*

**[00115]** Two factors have led us to explore internal fragments of VEGF-D for enhanced activity. Firstly, it is the central region of VEGF-D which exhibits amino acid homology with all other members of the VEGF family. Secondly, proteolytic processing which gives rise to internal bioactive polypeptides occurs for other growth factors such as PDGF-BB. In addition, the activity seen with the full length VEGF-D protein in COS cells was lower than for the corresponding conditioned medium from VEGF-A transfected COS cells.

**[00116]** It was predicted that the mature VEGF-D sequence would be derived from a fragment contained within residues 92-205, with cleavage at FAA^TFY and IIRR^SIQI. Immunoprecipitation analysis of VEGF-D*full*FLAG expressed in COS cells produced species consistent with the internal proteolytic cleavage of the VEGF-D polypeptide at these sites. Therefore, a truncated form of VEGF-D, with the N- and C-terminal regions removed (VEGFDΔNΔC), was produced and expressed in COS cells. This protein was identified and purified using the M2 antibody. The VEGFDΔNΔC protein was also detected by the A2 antibody, which recognizes a peptide within the 92-205 fragment of VEGF-D (not shown).

VEGFDΔNΔC was evaluated by the VEGFR2 bioassay and the Miles vascular permeability assay, and shown to bind to and activate the VEGFR2 receptor in a bioassay designed to detect cross-linking of the VEGFR2 extracellular domain. Induction of vascular permeability by this polypeptide in a Miles assay was at best marginal, in contrast to the effect of VEGF-A.

Example 10 VEGF-D Binds to and Activates VEGFR-3

**[00117]** The human VEGF-D cDNA was cloned into baculovirus shuttle vectors for the production of reconmbinant VEGF-D. In addition to baculoviral shuttle vectors, which contained the unmodified VEGF-D cDNA (referred to as "full length VEGF-D") two baculoviral shuttle vectors were assembled, in which the VEGF-D cDNA was modified in the following ways.

**[00118]** In one construct (referred to as "full length VEGF-D-$H_6$") a C-terminal histidine tag was added. In the other construct the putative N- and C-terminal propeptides were removed, the melittin signal peptide was fused in-frame to the N-terminus, and a histidine tag was added to the C-terminus of the remaining VEGF homology domain (referred to as "Δ NΔc-MELsp-VEGF-D-$H_6$").

**[00119]** For each of the three constructs, baculoviral clones of two or three independent transfections were amplified. The supernatant of High Five (HF) cells was harvested 48 h post infection with high ~~titer~~ titer virus stocks. The supernatant was adjusted to pH 7 with NaOH and diluted with one volume of D-MEM (0.2% FCS).

**[00120]** The samples were tested for their ability to stimulate tyrosine phosphorylation of VEGFR-3 ( ~~Flt4~~ Flt-4 receptor) on NIH3T3 cells, as described by Joukov *et al.*, 1996. The supernatant of uninfected cells and the supernatant of cells infected with the short splice variant of VEGF-C, which does not stimulate tyrosine

phosphorylation of VEGFR-3, were used as negative controls. VEGF-C modified in the same way as ΔNΔC-melSP-VEGF-D-$H_6$ was used as positive control. The results are shown in Figure 14.

**[00121]** The appearance of new bands at 125 and 195 kD indicates phosphorylation, and hence activation, of the receptor.

Example 11 VEGF-D Binds to and Activates VEGFR-2

**[00122]** Modified and unmodified human VEGF-D cDNA was cloned into baculovirus shuttle vectors for the production of recombinant VEGF-D as described in Example 10.

**[00123]** For each of the three constructs full length VEGF-D, full length VEGF-D-$H_6$, and ΔNΔC-melSP-VEGF-D-$H_6$, baculoviral clones of two or three independent transfections were amplified. The supernatant of High Five (HF) cells was harvested 48 hours post infection with high ~~titer~~ titer virus stocks. The supernatant was adjusted to pH 7 with NaOH and diluted with one volume of D-MEM (0.2% FCS).

**[00124]** The supernatants conditioned with the histidine-tagged proteins were tested for their ability to stimulate tyrosine phosphorylation of the KDR receptor according to Joukov *et al.*, 1996. KDR is the human homolog ~~homologue~~ of flk1 (VEGFR-2).

**[00125]** The supernatant of uninfected cells and the supernatant of cells infected with the VEGF-C 156S mutant, which does not stimulate KDR, were used as negative controls. $VEGF_{165}$ and VEGF-C modified in the same way as ΔNΔC-melSP-VEGF-D-$H_6$ were used as positive controls. The results are shown in Figure 15.

**[00126]** The appearance of a new band at approximately 210 kD indicates phosphorylation, and hence activation, of the receptor.

Example 12 Analysis of VEGF-D Gene Expression

**[00127]** In order to characterize ~~characterise~~ the pattern of VEGF-D gene expression in the human and in mouse embryos, VEGF-D cDNAs were used as hybridization probes for Northern blot analysis of polyadenylated human RNA and for *in situ* hybridization analysis with mouse embryos.

*Gene expression in the adult human*

**[00128]** A 1.1 kb fragment of the human VEGF-D cDNA shown in Figure 4 (~~SEQ ID NO.~~ SEQ ID NO:4) spanning from the *Eco*RV site to the 3'-terminus (nucleotides 911 to 2029) was ~~labelled~~ labeled with [$\alpha$-$^{32}$P]dATP using the Megaprime DNA labeling ~~labelling~~ system (Amersham) according to manufacturer's instructions. This probe was used to screen human multiple tissue northern blots (Clontech) by hybridization, also according to manufacturer's instructions. These blots contained polyadenylated RNA obtained from tissues of adult humans who were apparently free of disease. Autoradiography with the ~~labelled~~ labeled blots revealed that VEGF-D mRNA was most abundant in heart, lung, and skeletal muscle. VEGF-D mRNA was of intermediate abundance in spleen, ovary, small intestine, and colon, and was of low abundance in kidney, pancreas, thymus, prostate, and testis. No VEGF-D mRNA was detected in RNA from brain, placenta, liver, or peripheral blood leukocytes. In most of the tissues where VEGF-D mRNA was detected the size of the transcript was 2.3 kb. The only exception was skeletal muscle, where two VEGF-D transcripts of 2.3 kb and 2.8 kb were detected. In skeletal muscle the 2.3 kb transcript was more abundant than the 2.8 kb transcript.

*Gene expression in mouse embryos*

[00129] In order to generate an antisense RNA probe for mouse VEGF-D mRNA, the mouse VEGF-D2 cDNA shown in Figure 7 (~~SEQ ID NO.~~ SEQ ID NO:7) was inserted into the transcription vector pBluescriptIIKS+ (Stratagene). The resulting plasmid was digested to completion with the restriction endonuclease *Fok*I and then used as template for an *in vitro* transcription reaction with T3 RNA polymerase. This transcription reaction gave rise to an antisense RNA probe for VEGF-D mRNA which was complementary in sequence to the region of the VEGF-D2 cDNA (Figure 7) from the 3'-terminus to the *Fok*I cleavage site closest to the 3'-terminus (nucleotides 1135 to 700). This antisense RNA probe was hybridized at high stringency with paraffin-embedded tissue sections generated from mouse embryos at post-coital day 15.5. Hybridization and washing were essentially as described previously (Achen *et al.*, 1995).

[00130] After washing and drying, slides were exposed to autoradiography film for six days.

[00131] Development of the autoradiography film revealed that VEGF-D mRNA is localized ~~localised~~ in the developing lung of post-coital day 15.5 embryos. The signal for VEGF-D mRNA in the lung was strong and highly specific. Control hybridizations with sense probe gave no detectable background in lung or any other tissue.

*Summary*

[00132] The VEGF-D gene is broadly expressed in the adult human, but is certainly not ubiquitously expressed. Strongest expression was detected in heart, lung and skeletal muscle. In mouse embryos at post-coital day 15.5, strong and specific expression of the VEGF-D gene was detected in the lung. These data suggest that VEGF-D may play a role in lung development, and that expression

of the VEGF-D gene in lung persists in the adult, at least in humans. Expression of the gene in other tissues in the adult human suggests that VEGF-D may fulfill other functions in other adult tissues.

Example 13 VEGF-D is Mitogenic for Endothelial Cells

**[00133]** Some members of the VEGF family of proteins, namely VEGF-A (Leung *et al.*, 1989) and VEGF-B (Olofsson *et al.*, 1996), are mitogenic for endothelial cells. In order to test the mitogenic capacity of VEGFDΔNΔC for endothelial cells, this protein was expressed and purified by affinity chromatography as described in Example 9. Fraction #3, eluted from the M2 affinity column, which contained VEGFDΔNΔC, was diluted 1 in 10 in cell culture medium containing 5% serum and applied to bovine aortic endothelial cells (BAEs) which had been propagated in medium containing 10% serum. The BAEs had been seeded in 24-well dishes at a density of 10,000 cells per well the day before addition of VEGFDΔNΔC, and 3 days after addition of this polypeptide the cells were dissociated with trypsin and counted. Purified VEGF-A was included in the experiment as positive control. Results are shown in Figure 16. The addition of fraction #3 to the cell culture medium led to a 2.4-fold increase in the number of BAEs after 3 days of incubation, a result which was comparable to that obtained with VEGF-A. Clearly VEGFDΔNΔC is mitogenic for endothelial cells.

Example 14 Localization of the VEGF-D Gene on Human Chromosomes

**[00134]** In order to generate hybridization probes for localization of the VEGF-D gene on human chromosomes, a human genomic DNA clone for VEGF-D was isolated from a human genomic DNA library (Clontech). The genomic library was screened by hybridization with the human VEGF-D cDNA

shown in Figure 4, using standard methods (Sambrook *et al.*, 1989). One of the clones thus isolated was shown to contain part of the VEGF-D gene by hybridization to numerous oligonucleotides which were derived in sequence from the human VEGF-D cDNA. A region of the genomic clone, approximately 13 kb in size, was purified from agarose gel, labeled ~~labelled~~ by nick-translation with biotin-14-dATP and hybridized *in situ* at a final concentration of 20 ng/µl to metaphases from two normal human males. The fluorescence *in situ* hybridization (FISH) method was modified from that previously described (Callen *et al.*, 1990) in that chromosomes were stained before analysis with propidium iodide (as counterstain) and DAPI (for chromosome identification). Images of metaphase preparations were captured by a cooled CCD camera, using the CytoVision Ultra image collection and enhancement system (Applied Imaging Int. Ltd.). FISH signals and the DAPI banding pattern were merged for analysis.

**[00135]** Fifteen metaphases from the first normal male were examined for fluorescent signal. Ten of the metaphases showed signal on one chromatid (3 cells) or both chromatids (7 cells) of the X chromosome in band p22.1. There was a total of 9 non-specific background dots observed in these 15 metaphases. A similar result was obtained from hybridization of the probe to 15 metaphases from the second normal male, where signal was observed at Xp22.1 on one chromatid in 7 cells and on both chromatids in 4 cells. In conclusion, the human VEGF-D gene is located on the X chromosome in band p22.1.

Example 15 Localization of the murine VEGF-D Gene on Mouse Chromosomes

**[00136]** The mouse chromosomal location of the VEGF-D gene was determined by interspecific backcross analysis using progeny generated by mating (C57BL/6J x *Mus spretus*)F1 females and CB7BL/67 males as described previously (Copeland and Jenkins, 1991). This interspecific backcross mapping panel has been typed for over 2400 loci that are well distributed among all the autosomes as well as the X chromosome (Copeland and Jenkins, 1991). C57BL/6J and *M. spretus* DNAs were digested with several enzymes and analyzed by Southern blot hybridization for informative restriction fragment length polymorphisms (RFLPs) using a 1.3 kb mouse VEGF-D cDNA probe essentially as described (Jenkins *et al.* 1982). Fragments of 7.1, 6.3, 4.7, 2.5 and 2.2 kb were detected in *Taq*I-digested C57BL/6J DNA and major fragments of 7.1, 3.7, 2.7 and 2.2 kb were detected in *Taq*I-digested *M. spretus* DNA. The presence or absence of the 3.7 and 2.7 *Taq*I *M. spretus*-specific fragments, which cosegregated, was followed in backcross mice. The mapping results indicated that the VEGF-D gene is located in the distal region of the mouse X chromosome linked to *Bik, DxPasI* and *Ptmb4*. Although 89 mice were analyzed for every marker, up to 133 mice were typed for some pairs of markers. Each locus was analyzed in pairwise combinations for recombination frequencies using the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the most likely gene order are: centromere - *Btk* - 14/121 - *DxPasI* - 3/99 - VEGF-D - 5/133 - *Ptmb4*. The recombination frequencies [expressed as genetic distances in centiMorgans (cM) $\pm$ $\pm$ the standard error], calculated using Map Manager (version 2.6.5), are - *Btk* - 11.6+/-2.9 - *DxPasI* - 3.0+/-1.7 - VEGF-D - 3.8+/-1.7 - *Ptmb4*. A description of the probes and RFLPs for the loci linked to the VEGF-D gene, including *Btk, DxPasI*

and *Ptmb4*, has been reported previously (Hacfliger *et al.*, 1992; Holloway *et al.*, 1997).

**[00137]** We have compared our interspecific map of the X chromosome with a composite mouse linkage map that reports the map location of many uncloned mutations (provided from Mouse Genome Database, a computerized database maintained at The Jackson Library, Bar Harbor, ME). The VEGF-D gene mapped in a region of the composite map that lacks mouse mutations with a phenotype that might be expected for an alteration in the locus for an endothelial cell mitogen. The distal region of the mouse X-chromosome shares a region of homology with the short arm of the human X chromosomes (Mouse Genome Database). The placement of the VEGF-D gene in this interval in mouse suggests that the human homolog will map to Xp22. This is consistent with our FISH analysis which has localized the human gene to Xp22.1.

**[00138]** Numerous disease states are caused by mutations in unknown genes which have been mapped to Xp22.1 and the positions immediately surrounding this region in the human. These disease states include Kallmann syndrome, ocular albinism (Nettleship-Falls type), ocular albinism and sensorineural deafness, Partington syndrome, spondyloepiphyseal dysplasia (late), retinitis pigmentosa 15, gonadal dysgenesis (XY female type), Nance-Horan cataract-dental syndrome, retinoschisis, Charcot-Marie-Tooth disease, F-cell production, hypomagnesemia, keratosis follicularis spinulosa decalvans, Coffin-Lowry syndrome, corneal dermoids, hypophosphatemia, agammaglobulinemia, Aicardi symdrome, hereditary hypophosphatemia II, mental retardation (non-dysmorphic), Opitz G syndrome, pigment disorder (reticulate), dosage-sensitive sex reversal, adrenal hypoplasia, retinitis pigmentosa-6, deafness 4 (congenital sensorineural) and Wilson-Turner syndrome. The positions of the genes

involved in these disease states are documented in the OMIM gene map which is edited by Dr. Victor McKusick and colleagues at Johns Hopkins University (USA).

BIOASSAYS TO DETERMINE THE FUNCTION OF VEGF-D

**[00139]** Other assays are conducted to evaluate whether VEGF-D has similar activities to VEGF in relation to endothelial cell function, angiogenesis and wound healing. Further assays may also be performed, depending on the results of receptor binding distribution studies.

**I. Assays of Endothelial Cell Function**

a) *Endothelial cell proliferation*

**[00140]** Endothelial cell growth assays are performed by methods well known in the art, *e.g.*, those of Ferrara & Henzel (1989), Gospodarowicz *et al.* (1989), and/or Claffey *et al.*, Biochim. Biophys. Acta, 1995 1246 1-9.

b) *Cell adhesion assay*

**[00141]** The effect of VEGF-D on adhesion of polmorphonuclear granulocytes to endothelial cells is tested.

c) *Chemotaxis*

**[00142]** The standard Boyden chamber chemotaxis assay is used to test the effect of VEGF-D on chemotaxis.

d) *Plasminogen activator assay*

**[00143]** Endothelial cells are tested for the effect of VEGF-D on plasminogen activator and plasminogen activator inhibitor production, using the method of Pepper *et al.* (1991).

e) *Endothelial cell Migration assay*

**[00144]** The ability of VEGF-D to stimulate endothelial cells to migrate and form tubes is assayed as described in Montesano *et al.* (1986). Alternatively, the three-dimensional collagen gel assay described by Joukov *et al.* (1996) or a gelatinized membrane in a modified Boyden chamber (Glaser *et al.*, 1980) may be used.

**II Angiogenesis Assay**

**[00145]** The ability of VEGF-D to induce an angiogenic response in chick chorioallantoic membrane is tested as described in Leung *et al.* (1989). Alternatively the rat cornea assay of Rastinejad *et al.* (1989) may be used; this is an accepted method for assay of *in vivo* angiogenesis, and the results are readily transferrable to other *in vivo* systems.

**III Wound Healing**

**[00146]** The ability of VEGF-D to stimulate wound healing is tested in the most clinically relevant model available, as described in Schilling *et al.* (1959) and utilized ~~utilised~~ by Hunt *et al.* (1967).

**IV The Haemopoietic System**

**[00147]** A variety of *in vitro* and *in vivo* assays using specific cell populations of the haemopoietic system are known in the art, and are outlined below. In particular a variety of *in vitro* murine stem cell assays using fluorescence-activated cell sorter purified cells are particularly convenient:

a) *Repopulating Stem Cells*

**[00148]** These are cells capable of repopulating the bone marrow of lethally irradiated mice, and have the $Lin^-$, $Rh^{h1}$, $Ly\text{-}6A/E^+$, $c\text{-}kit^+$ phenotype. VEGF-D is tested on these cells either alone, or by co-incubation with other factors, followed by measurement of cellular proliferation by $^3H$-thymidine incorporation.

b) *Late Stage Stem Cells*

**[00149]** These are cells that have comparatively little bone marrow repopulating ability, but can generate D13 CFU-S. These cells have the $Lin^-$, $Rh^{h1}$, $Ly\text{-}6A/E^+$, $c\text{-}kit^+$ phenotype. VEGF-D is incubated with these cells for a period of time, injected into lethally irradiated recipients, and the number of D13 spleen colonies enumerated.

c) *Progenitor-Enriched Cells*

**[00150]** These are cells that respond *in vitro* to single growth factors and have the $Lin^-$, $Rh^{h1}$, $Ly\text{-}6A/E^+$, $c\text{-}kit^+$ phenotype. This assay will show if VEGF-D can act directly on haemopoietic progenitor cells. VEGF-D is incubated with these cells in agar cultures, and the number of colonies present after 7-14 days is counted.

## V Atherosclerosis

**[00151]** Smooth muscle cells play a crucial role in the development or initiation of atherosclerosis, requiring a change of their phenotype from a contractile to a synthetic state. Macrophages, endothelial cells, T lymphocytes and platelets all play a role in the development of atherosclerotic plaques by influencing the growth and phenotypic modulations of smooth muscle cell.

An *in vitro* assay using a modified Rose chamber in which different cell types are seeded on to opposite coverslips measures the proliferative rate and phenotypic modulations of smooth muscle cells in a multicellular environment, and is used to assess the effect of VEGF-D on smooth muscle cells.

**VI Metastasis**

[00152] The ability of VEGF-D to inhibit metastasis is assayed using the Lewis lung carcinoma model, for example using the method of Cao *et al.* (1995).

**VII VEGF-D in Other Cell Types**

[00153] The effects of VEGF-D on proliferation, differentiation and function of other cell types, such as liver cells, cardiac muscle and other cells, endocrine cells and osteoblasts can readily be assayed by methods known in the art, such as $^{3}$H-thymidine uptake by *in vitro* cultures. Expression of VEGF-D in these and other tissues can be measured by techniques such as Northern blotting and hybridization or by *in situ* hybridization.

**VIII Construction of VEGF-D Variants and Analogs ~~Analogues~~**

[00154] VEGF-D is a member of the PDGF family of growth factors which exhibits a high degree of homology to the other members of the PDGF family. VEGF-D contains eight conserved cysteine residues which are characteristic of this family of growth factors. These conserved cysteine residues form intra-chain disulfide bonds which produce the cysteine knot structure, and inter-chain disulfide bonds that form the protein dimers which are characteristic of members of the PDGF family of growth

factors. VEGF-D will interact with protein tyrosine kinase growth factor receptors.

**[00155]** In contrast to proteins where little or nothing is known about the protein structure and active sites needed for receptor binding and consequent activity, the design of active mutants of VEGF-D is greatly facilitated by the fact that a great deal is known about the active sites and important amino acids of the members of the PDGF family of growth factors.

**[00156]** Published articles elucidating the structure/activity relationships of members of the PDGF family of growth factors include for PDGF: Oestman *et al.*, J. Biol. Chem., 1991 266 10073-10077; Andersson *et al.*, J. Biol. Chem., 1992 267 11260-1266; Oefner *et al.*, EMBO J., 1992 11 3921-3926; Flemming *et al.*, Molecular and Cell Biol., 1993 13 4066-4076 and Andersson *et al.*, Growth Factors, 1995 12 159-164; and for VEGF: Kim *et al.*, Growth Factors, 1992 7 53-64; Pötgens *et al.*, J. Biol. Chem., 1994 269 32879-32885 and Claffey *et al.*, Biochem. Biophys. Acta, 1995 1246 1-9. From these publications it is apparent that because of the eight conserved cysteine residues, the members of the PDGF family of growth factors exhibit a characteristic knotted folding structure and dimerization, which result in formation of three exposed loop regions at each end of the dimerized molecule, at which the active receptor binding sites can be expected to be located.

**[00157]** Based on this information, a person skilled in the biotechnology arts can design VEGF-D mutants with a very high probability of retaining VEGF-D activity by conserving the eight cysteine residues responsible for the knotted folding arrangement and for dimerization, and also by conserving, or making only conservative amino acid substitutions in the likely receptor sequences in the loop 1, loop 2 and loop 3 region of the protein structure.

**[00158]** The formation of desired mutations at specifically targeted sites in a protein structure is considered to be a standard technique in the arsenal of the protein chemist (Kunkel *et al.*, Methods in Enzymol., 1987 154 367-382). Examples of such site-directed mutagenesis with VEGF can be found in Pötgens *et al.*, J. Biol. Chem., 1994 269 32879-32885 and Claffey *et al.*, Biochim. Biophys. Acta, 1995 1246 1-9. Indeed, site-directed mutagenesis is so common that kits are commercially available to facilitate such procedures (*e.g.* Promega 1994-1995 Catalog., Pages 142-145).

**[00159]** The endothelial cell proliferating activity of VEGF-D mutants can be readily confirmed by well established screening procedures. For example, a procedure analogous to the endothelial cell mitotic assay described by Claffey *et al.*, (Biochim. Biophys. Acta., 1995 1246 1-9) can be used. Similarly the effects of VEGF-D on proliferation of other cell types, on cellular differentiation and on human metastasis can be tested using methods which are well known in the art.

**[00160]** It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

**[00161]** References cited herein are listed on the following pages, and are incorporated herein by ~~this~~ reference.

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